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High-affinity binding of β-carbolines to imidazoline I_{2B} receptors and MAO-A in rat tissues: Norharman blocks the effect of morphine withdrawal on DOPA/noradrenaline synthesis in the brain

Antonio Miralles, Susana Esteban, Antonio Sastre-Coll, David Moranta, Víctor José Asensio, Jesús Andrés García-Sevilla *

Laboratori de Neurofarmacologia, Institut Universitari d'Investigació en Ciències de la Salut (IUNICS), Universitat de les Illes Balears, Ctra. Valldemossa km 7.5, E-07122, Palma de Mallorca, Spain

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Abstract

This study was designed to determine the affinity and binding profile of β-carbolines for imidazoline I_2 receptors and catalytic sites of monoamine oxidase (MAO)-A/B in rat brain and liver. The aim was also directed to assess the in vivo effects of norharman (β-carboline) and LSL 60101 (I_2 ligand) on brain 3,4-dihydroxyphenylalanine (DOPA) synthesis in morphine-dependent rats. Competition experiments against [^{3}H]2-BFI revealed that β-carbolines recognize the high- and low-affinity components of the brain imidazoline I_2 receptor with the rank order of potency (K_{iH} in nM): noreleagnine (12)>norharman (20)>harmalol (82)>harmaline (177) ≫ harmine (630)>harman (700) ≫ FG-7142 (>100,000). In liver, this rank was different: harmine (51)>harmaline (103)=noreleagnine (103) ≫ harmalol (1290)>harman (2000) ≫ norharman (12,382) ≫ FG-7142 (>100,000). In brain and liver, competition curves for β-carbolines against [^{3}H]Ro41-1049 (MAO-A) and [^{3}H]Ro19-6327 (MAO-B) were monophasic and resulted in different drug potencies for the two MAO isozymes (higher affinities for MAO-A) and in similar pharmacological profiles in both tissues. In morphine-dependent rats, naloxone (2 mg/kg, 2 h)-precipitated withdrawal increased the synthesis of DOPA in the cerebral cortex and hippocampus (50%). Pretreatment with norharman (20 mg/kg) or LSL 60101 (20 mg/kg) (30 min before naloxone) fully prevented the stimulatory effect of opiate withdrawal on DOPA synthesis. Norharman and LSL 60101 also attenuated the severity of the withdrawal syndrome. The results indicate that β-carbolines bind with high affinity to imidazoline I_{2B} receptors, and similarly to I_2 ligands (LSL 60101) can block the behavioural and biochemical effects of opiate withdrawal.

Keywords: β-carboline; Norharman; Imidazoline I_2 receptor; [^{3}H]2-BFI; [^{3}H]Ro41-1049; [^{3}H]Ro19-6327 (lazabemide); Morphine addiction; (Rat) brain and liver

1. Introduction

The presence of imidazoline receptors in various tissues, including the central nervous system (CNS), is now well established (Regunathan and Reis, 1996; Eglen et al., 1998). These receptors have been separated into three entities, based on pharmacological and functional criteria: a clonidine-preferring site (imidazoline I_1 receptor, involved in the control of blood pressure; Bousquet et al., 2003), an idazoxan-preferring site (imidazoline I_2 receptor, modulated in various psychiatric disorders; García-Sevilla et al., 1999), and an atypical imidazoline site found on pancreatic β cells (imidazoline I_3 receptor, involved in the control of insulin secretion; Morgan and Chan, 2001). The imidazoline I_2 receptor has been further divided into the I_{2A} subtype (e.g. human placenta) and I_{2B} subtype (e.g. rat brain), based on the affinity displayed by the guanidide amiloride (Miralles et al., 1993; Olmos et al., 1996). Since the demonstration that

* Corresponding author. Tel.: +34 971 17 31 48; fax: +34 971 17 31 84.
E-mail address: jesus.garcia-sevilla@uib.es (J.A. García-Sevilla).

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imidazoline I_2 binding sites also are located on the outer membrane of mitochondria (Tesson et al., 1991), several studies have related these receptors with the enzyme monoamine oxidase (MAO). Thus, imidazoline compounds were shown to inhibit MAO-A/B isoforms through competitive interactions (Carpéné et al., 1995; Ozaita et al., 1997), and chronic treatments with MAO inhibitors reduced the density of I_2 sites in rat tissues (Olmos et al., 1993; Alemany et al., 1997). Later it was reported that I_2 sites in liver and brain were lost in MAO-B, but not MAO-A, deficient mice (Remaury et al., 2000), and that MAO inhibition by imidazoline ligands could occur through allosteric mechanisms (Raddatz et al., 2000).

Recently, various β-carbolines were shown to bind with high affinity and selectivity to imidazoline I_1, I_2 and/or I_3 receptors (Husbands et al., 2001; Morgan et al., 2003; Glennon et al., 2004). Since some β-carbolines (e.g. harman and norharman) also occur endogenously in mammalian tissues (Fekkes and Bode, 1993), it was proposed that these compounds could represent endogenous ligands for imidazoline-binding proteins (Robinson et al., 2003). In addition, most β-carbolines are potent and reversible inhibitors of MAO, being more selective for MAO-A than MAO-B (Glover et al., 1982; Rommelspacher et al., 1994; Kim et al., 1997), and could modulate monoaminergic neurotransmission in the CNS (Iurlo et al., 2001).

Imidazoline receptor ligands and β-carbolines share in common the ability to interact with the opioid system in the CNS (García-Sevilla et al., 1999; Robinson et al., 2003). Notably, agmatine and I_2 receptor ligands were shown to attenuate the development of tolerance to opiate drugs (Kolesnikov et al., 1996; Boronat et al., 1998) and to enhance supraspinal morphine analgesia (Sánchez-Blazquez et al., 2000) in rodents. On the other hand, the involvement of the noradrenergic system in the expression of the somatic symptoms of opiate withdrawal is well documented (review in Sastre-Coll et al., 2002). Thus, I_2 receptor ligands (2-BFI and BU224) and/or β-carbolines (norharman, harman and harmine) also were effective in attenuating some behavioural (Cappendijk et al., 1994; Hudson et al., 1999; Aricioglu-Kartal et al., 2003) and electrophysiological (Ruiz-Durántez et al., 2003) effects induced by opiate withdrawal in morphine-dependent rats.

In this context, this study aimed to determine the affinity and binding profile of a series of β-carbolines for imidazoline I_2 receptors in rat brain and liver, as compared with those for the catalytic sites of MAO-A/B isoforms in the same tissues. Moreover, the aim was also directed to assess in vivo the effect of the β-carboline norharman and the imidazoline I_2 receptor ligand LSL 60101 on the up-regulated activity of the enzyme tyrosine hydroxylase (synthesis of DOPA/noradrenaline) induced by opiate withdrawal in morphine-dependent rats (Sastre-Coll et al., 2002).

2. Materials and methods

2.1. Animals, tissues and treatments

Adult male Sprague–Dawley rats (250–300 g) were used. The animals received a standard diet with water freely available and were housed at 20±2 °C with a 12 h light/dark cycle. For radioligand binding experiments, naive rats were decapitated and the parieto-occipital cortex and portions of liver were rapidly removed into ice-cold Tris–sucrose buffer (5 mM Tris–HCl, 250 mM sucrose, 1 mM $MgCl_2$, pH 7.4) and then frozen at −80 °C until required. For the in vivo experiments on the synthesis of DOPA/noradrenaline, the brains of control and treated rats were dissected on an ice-cold plate into the parieto-occipital cortex and hippocampus and processed as described below. For the acute drug treatments, the rats received a single intraperitoneal (i.p.) injection of 0.9% saline (1 ml/kg, i.p.), morphine (30 mg/kg, 1 h), norharman (20 mg/kg, 1 h) or LSL 61101 (20 mg/kg, 1 h). After this injection (30 min) the animals received the aromatic L-amino acid decarboxylase inhibitor NSD 1015 (3-hydroxybenzylhydrazine, 100 mg/kg) and then were sacrificed after another 30 min. For the chronic morphine treatments (opiate tolerance and dependence), the rats were injected i.p. three times daily during five consecutive days with increasing doses of the opiate (10–100 mg/kg) as described previously (Sastre-Coll et al., 2002). Control rats were similarly treated with 0.9% saline. The withdrawal syndrome in morphine-dependent rats was precipitated by naloxone (2 mg/kg, i.p. 2 h), resulting in various withdrawal reactions (e.g. abnormal posturing, ptosis, diarrhea, penile erection, weight loss, wet-dog shakes, teeth-chattering) that were checked and counted for each rat when present during a 30-min period, and its severity was finally expressed as a composite score (for details see Gabilondo and García-Sevilla, 1995). Other groups of rats received a single i.p. injection of 0.9% saline, norharman (20 mg/kg) or LSL 60101 (20 mg/kg) 30 min before the last administration of saline, morphine or naloxone (chronic treatments), and then the animals received NSD 1015 as above and were sacrificed after another 30 min. Three independent groups of rats were prepared for behavioural experiments (i.e. chronic morphine plus naloxone; chronic morphine plus norharman plus naloxone; chronic morphine plus LSL 60101 plus naloxone). This study was approved by the research review board of the Dirección General de Investigación (MEC, Madrid), and the experiments in rats followed the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) and were performed according to the guidelines of the University of the Balearic Islands.

2.2. [^{3}H]2-BFI, [^{3}H]Ro 41-1049 and [^{3}H]Ro 19-6327 binding assays and analyses of binding data

The binding of [^{3}H]2-BFI (imidazoline I_2 receptors), [^{3}H]Ro 41-1049 (MAO-A) and [^{3}H]Ro 19-6327 (MAO-B) to brain and liver membranes (P_2 fractions) were assessed as described previously (Alemany et al., 1997; Ozaita et al., 1997). Briefly, the homogenized samples (Tris–sucrose buffer) were centrifuged at 1100 ×g for 10 min and the supernatants re-centrifuged at 40,000 ×g for 10 min. The resulting pellet was washed twice with the appropriate incubation buffer (for [^{3}H]2-BFI: 50 mM Tris–HCl, pH 7.5; for [^{3}H]Ro 41-1049 and [^{3}H]Ro 19-6327: 50 mM Tris–HCl, 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.5 mM EGTA, pH 7.4), and the final pellet resuspended in the corresponding buffer to a final protein content of 800–1000 μg/ml. For drug competition studies, 0.5 ml-aliquots of cortical or liver membranes were incubated with shaking

for 45 min at 25 °C with the radioligands (4×10^{-9} M) and in the absence or presence of various concentrations of the competing drugs (3×10^{-11} M to 10^{-3} M; 15–22 concentrations). Nonspecific binding was determined in the presence of 10^{-4} M naphazoline ([^{3}H]2-BFI), 10^{-6} M clorgyline ([^{3}H]Ro 41-1049) or 10^{-4} M deprenyl ([^{3}H]Ro 19-6327). The incubations were stopped by diluting the samples with of ice-cold Tris incubation buffer. Bound and free radioligands were separated by vacuum filtration through Whatman GF/C glass fibre filters, which had been presoaked with 1% polyethylenimide, using a Brandel 48R cell harvester (Biomedical Research and Development Laboratories, USA). The filters were rinsed twice with Tris incubation buffer, air-dried, transferred to minivials containing 5 ml of OptiPhase 'HiSafe' II cocktail (LKB, England) and counted for radioactivity by liquid scintillation spectrometry at 57% efficiency (Packard model 1900 TR).

Analyses of competition experiments (K_i, inhibition constant) as well as the fitting of data to the appropriate binding model were performed by computer-assisted nonlinear analysis from untransformed data using the EBDA-LIGAND programs (for details see Alemany et al., 1997). All experiments were initially analysed assuming a one-site model of radioligand binding and then assuming a two-site binding model. The selection between the different binding models was made statistically using the extra sum of square principle (F test). The more complex model was accepted if the P value resulting from the F test was less than 0.05.

2.3. Tyrosine hydroxylase assay and analyses of HPLC data

The in vivo activity of tyrosine hydroxylase, rate-limiting enzyme in the pathway for the synthesis of catecholamines, was determined by measuring the accumulation of DOPA (3,4-dihydroxyphenylalanine) within 30 min after inhibition of the aromatic L-amino acid decarboxylase by a maximally effective dose of NSD 1015 (100 mg/kg, i.p.) (Carlsson et al., 1972; Nissbrandt et al., 1988). The DOPA formed from endogenous tyrosine was then determined by high-pressure liquid chromatography (HPLC) with electrochemical detection (ED), as described previously (Sastre-Coll et al., 2002).

Briefly, fresh brain regions enriched in noradrenaline (cerebral cortex and hippocampus) were weighed and homogenized in 0.4 M $HClO_4$, 0.01% K_2EDTA and 0.1% $Na_2S_2O_5$. The homogenate was then centrifuged at 40,000 $\times g$ for 15 min at 4 °C. The resulting supernatant was filtered through 0.45 μm syringe filters (Spartan-3, Aldrich Chemical, Milwaukee, WI, U.S.A.) and various aliquots

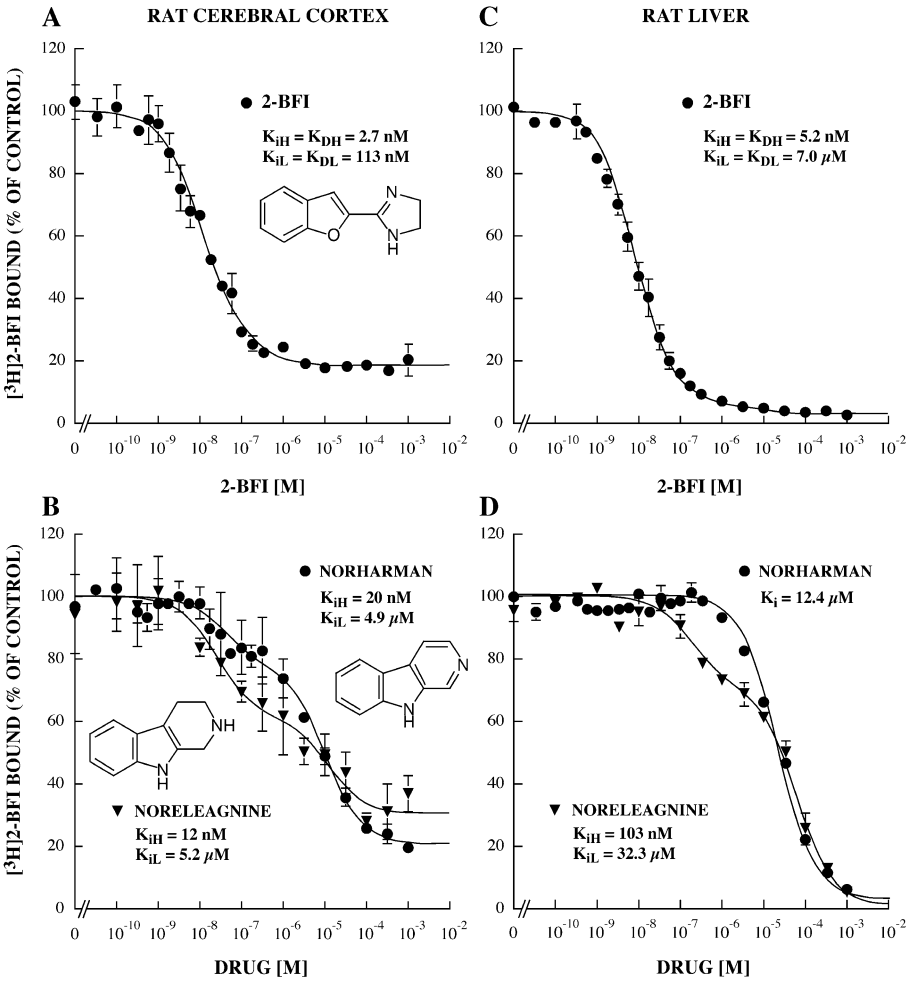


Fig. 1. Inhibition of binding of [^{3}H]2-BFI to rat brain cortical (A, B) and liver membranes (C, D) by 2-BFI and the β-carboline compounds norharman and noreleagnine. Data shown are the mean ± S.E.M. of three independent experiments for each drug. Computer-assisted curve fitting revealed that a two-site fit was significantly better than a one-site binding model ($P < 0.01$, F-test) for all drugs with the exception of norharman against [^{3}H]2-BFI in liver membranes (D). Binding parameters (K_i values) were determined directly by simultaneous analysis of three experiments for each drug using the EBDA-LIGAND programs.

were injected into the HPLC system (Spherisorb S3 ODS1 C18 reversed-phase column) for DOPA and noradrenaline determination. The compounds were detected electrochemically by means of a cell with a glassy carbon working electrode with an applied potential of +0.75 V against an Ag/AgCl reference electrode (Waters 460 Electrochemical Detector). The current produced was monitored using an interphase Waters bus SAT/IN Module connected to a digital PC. The concentrations of DOPA and noradrenaline in a given sample were calculated by interpolating the corresponding peak height into a parallel standard curve using the software Millennium[32] (Waters).

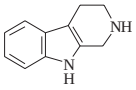
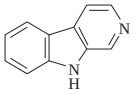
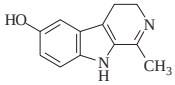
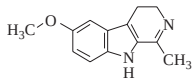
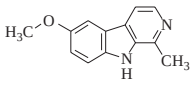
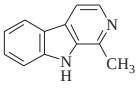
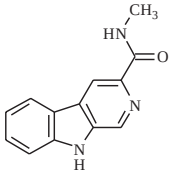
2.4. Data analyses and statistics

Results are expressed as means ± S.E.M. values. One-way analysis of variance (ANOVA) followed by Scheffé's multiple comparison test was used for the statistical evaluations (DOPA/noradrenaline synthesis). Behavioral data (nonparametric nature) were analyzed by Kruskal–Wallis one-way ANOVA followed by the Mann–Whitney *U*-test (Gabilondo and García-Sevilla, 1995). The level of significance was chosen as $P=0.05$.

2.5. Drugs and chemicals

[^{3}H]2-BFI (2-(2-benzofuranyl)-2-imidazoline) (specific activity 59 Ci/mmol) was purchased from Amersham International (Bucks, U.K.). [^{3}H]Ro 41-1049 (*N*-(2-amino-ethyl)-5-(*m*-fluorophenyl)-4-thiazole carboxamide) HCl (specific activity 31 Ci/mmol) and [^{3}H]Ro 19-6327 (lazabemide; *N*-(2-aminoethyl)-5-chloro-2-pyridine carboxamide) HCl (specific activity 20.2 Ci/mmol) were generous gifts from F. Hoffmann-La Roche Ltd., Basel, Switzerland. Other drugs (and their sources) included: 2-BFI (synthesized as LSL 61103 at Ipsen Pharma, S.A., Barcelona, Spain); FG-7142 (*N*-methyl-β-carboline-3-carboxamide) (Sigma Chemical Co., USA);

Table 1
Binding parameters of a series of β-carbolines to imidazoline I_2 receptors and MAO-A and -B isozymes in the rat cerebral cortex and liver membranes

Drug	Rat cerebral cortex					Rat liver				
	[^{3}H]2–BFI (imidazoline I_2 receptors)			[^{3}H]Ro41–1049 (MAO–A)	[^{3}H]Ro19–6327 (MAO–B)	[^{3}H]2–BFI (imidazoline I_2 receptors)			[^{3}H]Ro41–1049 (MAO–A)	[^{3}H]Ro19–6327 (MAO–B)
	K_{iH} (nM)	K_{iL} (nM)	% R_H	K_i (nM)	K_i (nM)	K_{iH} (nM)	K_{iL} (nM)	% R_H	K_i (nM)	K_i (nM)
Noreleagnine 	12	5197	55	355	11,700	103	32,340	30	495	15,990
Norharman 	20	4900	28	177	101		12,382	–	278	223
Harmalol 	82	21,409	32	32	155,500	1290	254,000	37	45	252,000
Harmaline 	177	5300	45	1.3	18,300	103	34,000	35	1.7	24,150
Harmine 	630	372,900	62	0.2	5141	51	5997	30	0.5	6420
Harman 	700	49,700	59	9.9	5890	2000	76,600	66	8.9	8240
FG–7142 		>100,000	–	2728	17,400		>100,000	–	2430	20,890

Rat brain cortical and liver membranes were incubated at 25 °C for 30 min with [^{3}H]-2BFI (4×10^{-9} M), [^{3}H]-Ro41-1049 (4×10^{-9} M) or [^{3}H]-Ro19-6327 (4×10^{-9} M) in the absence or presence of the competing drugs (3×10^{-11} to 10^{-3} M, 15–22 concentrations). Binding parameters (K_i values) were determined directly by simultaneous analysis of two to six independent experiments for each drug using the EBDA-LIGAND programs. A two-site fit was accepted only if it was significantly better than a one-site binding model. The proportion of receptors in high affinity (R_H) is given as per cent of the total number of receptors.

harmaline (1-methyl-7-methoxy-3,4-dihydro-β-carboline) HCl (Sigma); harmalol (1-methyl-7-hydroxy-3,4-dihydro-β-carboline) HCl (Sigma); harman (1-methyl-β-carboline) HCl (Sigma); harmine (1-methyl-7-methoxy-β-carboline) HCl (Sigma); LSL 60101 (2-(2-benzofuranyl)-2-imidazole) HCl (synthesized at Ipsen Pharma, S.A., U.K. Patent Application GB2 262 739 A); noreleagnine (1,2,3,4-tetrahydro-β-carboline) HCl (Sigma); norharman (β-carboline) HCl (Sigma); NSD 1015 (3-hydroxybenzyl-hydrazine, Sigma); Ro 19-6327 HCl and Ro 41-1049 HCl (F. Hoffmann-La Roche Ltd.). All other chemicals were from Sigma.

3. Results

3.1. Differential pharmacological profile of β-carbolines against the binding of [^{3}H]2-BFI in rat brain and liver

Homologous competition experiments with 2-BFI (a prototypical imidazoline I_2 receptor ligand) against [^{3}H]2-BFI binding to rat brain and liver membranes resulted in biphasic curves with slope factors slightly less than unity (n_H: 0.70–0.80). Computer-assisted nonlinear analysis best resolved these binding sites into high-and low-affinity components for 2-BFI in both tissues (Fig. 1A and C).

Competition experiments for a series of β-carbolines against [^{3}H]2-BFI binding to rat brain membranes were shallow and biphasic (e.g., see Fig. 1B for noreleagnine and norharman) with slope factors significantly less than unity (n_H values between 0.4–0.7). Curve fitting revealed that these compounds inhibited [^{3}H]2-BFI from two distinct binding sites, which indicated that these β-carbolines are able to recognize the high- and low-affinity components of the imidazoline I_2 receptor in the rat brain (Table 1 and Fig. 1B). The rank order of potency of β-carbolines competing with [^{3}H]2-BFI binding to rat brain membranes was (K_{iH} in nM): noreleagnine (12)>norharman (20)>harmalol (82)>harmaline (177) ≫ harmine (630)>harman (700) ≫ FG-7142 (>100,000) (Table 1).

In contrast, similar competition experiments with these drugs against [^{3}H]2-BFI binding to rat liver membranes resulted in a different rank order of potency (K_{iH} or K_{iL} in nM): harmine (51)> harmaline (103)=noreleagnine (103) ≫ harmalol (1290)>harman (2000) ≫ norharman (12,382) ≫ FG-7142 (>100,000) (Table 1; Fig. 1D). In this tissue, the competition of norharman against the radioligand resulted in a steep curve with a slope factor close to unity (n_H=0.98; Fig. 1D), whereas nonlinear analyses of competition curves for other β-carbolines (with the exception of FG-7142) indicated a significantly better fitting to a two-site binding model (n_H values between 0.4–0.7) (Table 1 and Fig. 1D).

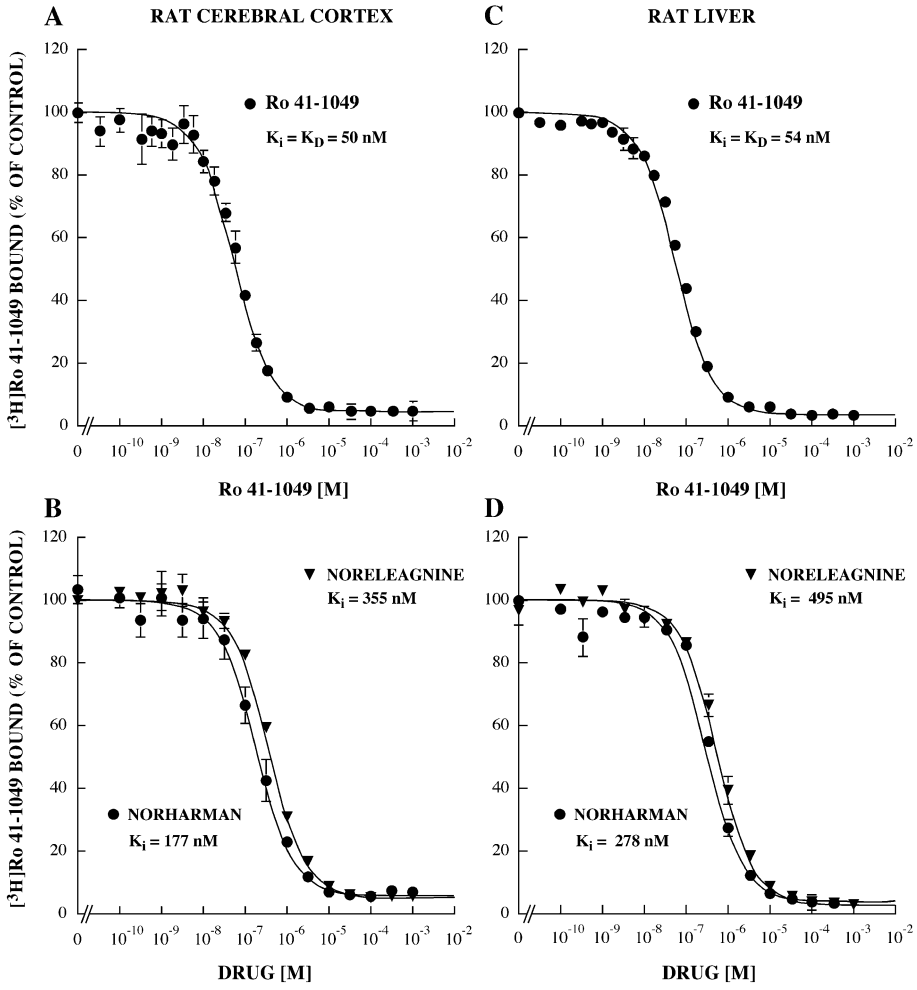


Fig. 2. Inhibition of binding of [^{3}H]Ro 41-1049 to rat brain cortical (A, B) and liver membranes (C, D) by Ro 41-1049 and the β-carboline compounds norharman and noreleagnine. Data shown are the mean±S.E.M. of three independent experiments for each drug. Binding parameters (K_i values) were determined directly by simultaneous analysis of three experiments for each drug using the EBDA-LIGAND programs.

To further assess the different pharmacological characteristics of the interaction of β-carbolines with imidazoline I_2 receptors in brain and liver, the various drug affinities (pK_i values) were compared in both tissues, which resulted in poor correlations (for pK_{iH} values: $r=0.22$, $P=0.68$; for pK_{iL} values: $r=0.45$, $P=0.45$).

3.2. Differential pharmacological profile of β-carbolines against the binding of [^{3}H]Ro 19-6327 and [^{3}H]Ro 41-1049 in rat brain and liver

Homologous competition experiments with Ro 41-1049 (MAO-A inhibitor) or Ro 19-6327 (MAO-B inhibitor) against the binding of the respective radioligand to rat brain and liver membranes resulted in monophasic curves (slope factors close to unity) with K_i values in the nanomolar range (Figs. 2A/C and 3A/C).

Competition curves for a series of β-carbolines against [^{3}H]Ro 41-1049 (MAO-A) and [^{3}H]Ro 19-6327 (MAO-B) binding to rat brain and liver membranes were steep and monophasic (slope factor close to unity), and resulted in different drug potencies for the two MAO isozymes and in similar pharmacological profiles in both tissues (Table 1; Figs. 2B/D and 3B/D). Thus, β-carbolines, with the exception of norharman, displayed higher affinities against [^{3}H]Ro 41-1049 than [^{3}H]Ro 19-6327 binding (Table 1), which confirmed that these compounds are potent MAO-A inhibitors. The rank order of potency of β-carbolines competing with [^{3}H]Ro 41-1049 binding to MAO-A in rat brain and liver membranes was (K_i in nM): harmine (0.2 and 0.5) > harmaline (1.3 and 1.7) > harman (9.9 and 8.9) > harmalol (32 and 45) > norharman (177 and 278) > noreleagnine (355 and 495) ≫ FG-7142 (2728 and 2430) (Table 1). In contrast, the rank order of potency of β-carbolines competing with [^{3}H]Ro 19-6327 binding to MAO-B in rat brain and liver membranes was (K_i in μM): norharman (0.1 and 0.2) ≫ harmine (5.1 and 6.4) ≈ harman (5.9 and 8.2) > noreleagnine (12 and 16) ≈ FG-7142 (17 and 21) ≈ harmaline (18 and 24) ≫ harmalol (156 and 252) (Table 1). Therefore and as expected, very good correlations resulted when the various drug affinities (pK_i values) in brain and liver for MAO-A sites ($r=0.99$, $P<0.0001$) ands MAO-B sites ($r=0.99$, $P<0.0001$) were compared.

3.3. Effects of acute treatments with norharman and LSL 60101 on brain tyrosine hydroxylase activity in morphine-dependent rats during naloxone-precipitated withdrawal

The acute administration of morphine (30 mg/kg, i.p., 1 h), but not that of norharman (20 mg/kg, i.p., 1 h) or LSL 60101 (20 mg/kg, i.p., 1 h), decreased the accumulation of DOPA in the cerebral

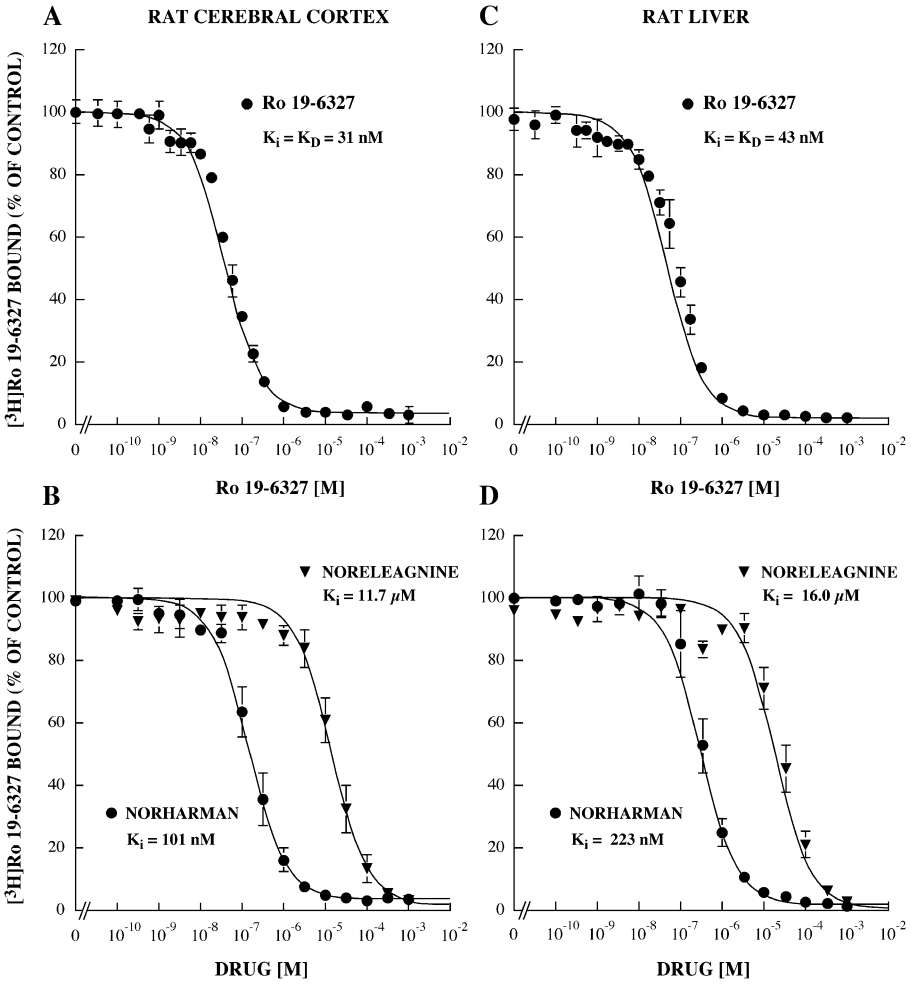


Fig. 3. Inhibition of binding of [^{3}H]Ro 19-6327 to rat brain cortical (A, B) and liver membranes (C, D) by Ro 19-6327 and the β-carboline compounds norharman and noreleagnine. Data shown are the mean ± S.E.M. of three independent experiments for each drug. Binding parameters (K_i values) were determined directly by simultaneous analysis of three experiments for each drug using the EBDA-LIGAND programs.

cortex (25%, $P<0.05$) and hippocampus (22%, $P<0.05$) (Fig. 4A). Morphine, norharman and LSL 60101 did not alter significantly the content of noradrenaline in these brain regions (data not shown). Chronic morphine treatment (10–100 mg/kg for 5 days) did not modify significantly the synthesis of DOPA in the cerebral cortex and hippocampus (Fig. 4B), indicating the induction of tolerance to the acute effect of the opiate. The acute treatment with norharman (20 mg/kg) and LSL 60101 (20 mg/kg) in morphine-tolerant rats did not alter significantly the accumulation of DOPA in both brain regions (Fig. 4B).

In morphine-dependent rats, naloxone (2 mg/kg, 2 h)-precipitated withdrawal significantly increased the synthesis of DOPA in the cerebral cortex (50%, $P<0.01$) and hippocampus (55%, $P<0.01$) (Fig. 4C). In these morphine-dependent rats, pretreatment with norharman (20 mg/kg) or LSL 60101 (20 mg/kg) (30 min before naloxone) almost fully prevented the stimulatory effect of opiate withdrawal on DOPA synthesis in both brain regions (Fig. 4C). Moreover, the severity of the withdrawal syndrome (composite behavioural score) was significantly reduced by norharman (control score: 21.3±3.0, $n=6$; norharman score: 12.2±3.5, $n=8$, $P<0.05$) and LSL 60101 (control score: 25.0±3.9, $n=5$; LSL score: 11.3±2.2, $n=10$, $P<0.02$).

4. Discussion

The present study provides further evidence that β-carbolines besides being able to bind to the catalytic site of MAO-A also bind with high affinity to imidazoline I_{2B} receptors in rat brain and liver membranes. Moreover, the β-carboline norharman and the selective imidazoline I_2 ligand LSL 60101 (Alemany et al., 1995) blocked the effect of morphine withdrawal on tyrosine hydroxylase activity in vivo (DOPA/noradrenaline synthesis) and attenuated the severity of the withdrawal reaction. This latter finding suggests that some endogenous β-carbolines (Cappendijk et al., 1994; Aricioglu-Kartal et al., 2003) and other I_2 ligands (Hudson et al., 1999) could modulate the symptoms of morphine withdrawal through the regulation of monoamine synthesis in the brain of morphine-dependent rats (cf. Sastre-Coll et al., 2002).

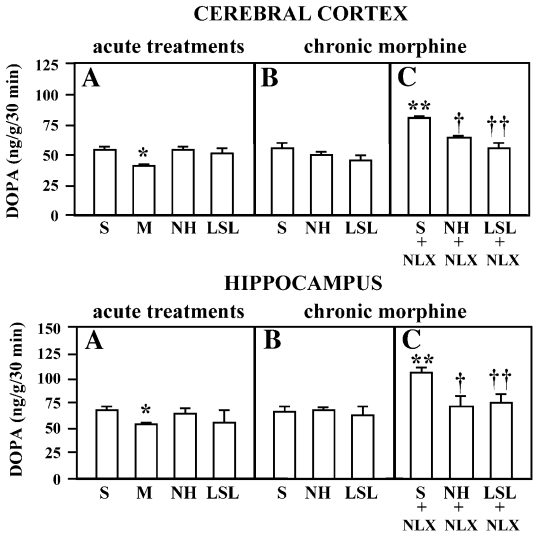


Fig. 4. (A) Acute effects of saline (S, 1 ml/kg i.p.), morphine (M, 30 mg/kg, i.p., 1 h), norharman (NH, 20 mg/kg, i.p., 1 h) and LSL 61101 (LSL, 20 mg/kg, i.p., 1 h) on DOPA accumulation (after inhibition of the aromatic L-amino acid decarboxylase) in the rat cerebral cortex and hippocampus. (B) Acute effects of saline, norharman and LSL 61101 on DOPA accumulation in chronic morphine-treated rats (10–100 mg/kg, i.p., 5 days). (C) Acute effects of saline, norharman and LSL 61101 on DOPA accumulation in naloxone (NLX)-precipitated opiate withdrawal in chronic morphine-treated rats. Columns are means±S.E.M. of 6–9 experiments for each treatment. *$P<0.05$, **$P<0.01$ when compared with the corresponding saline in (A) or (B). †$P<0.05$, ††$P<0.01$ when compared with saline plus naloxone in (C) (ANOVA followed by Scheffé's test).

Competition experiments of [^{3}H]2-BFI binding by 2-BFI in rat brain and liver membranes revealed a heterogeneous interaction with two binding sites for the radioligand, in agreement with studies in human brain (Callado et al., 2001), rabbit brain (Lione et al., 1996) and rat brain (Alemany et al., 1997). Moreover, most of the β-carbolines tested also displayed high affinity for the imidazoline I_{2B} receptor and recognized the low-affinity site of this receptor ([^{3}H]2-BFI binding in rat brain and liver), in general agreement with previous reports (Hudson et al., 1999; Husbands et al., 2001; Price et al., 1999). However, the drug-affinity profile obtained in the current study in the rat cerebral cortex (noreleagnine>norharman>harmalol>harmaline≫harmine>harman) differed from that described in rat whole brain (noreleagnine=harmine>harmaline>harman>norharman>harmalol) (Hudson et al., 1999; Husbands et al., 2001) and rabbit whole brain membranes (harman>harmaline>harmine>noreleagnine>norharman) (Price et al., 1999). In the present study, the rank order of potency of β-carbolines in rat liver membranes (harmine>harmaline=noreleagnine≫harmalol>harman≫norharman) was different to that found in the brain. These discrepancies in the affinity profiles for β-carbolines may be related to differences in brain region (cerebral cortex vs. whole brain), species (rat vs. rabbit) or tissues (brain vs. liver). In spite of these minor discrepancies the available data indicated that β-carbolines bind with high affinity to imidazoline I_2 receptors.

There is extensive evidence that both β-carbolines (Glover et al., 1982; Rommelspacher et al., 1994; Kim et al., 1997) and imidazoline compounds (Carpéné et al., 1995; Ozaita et al., 1997; Lalies et al., 1999) reversibly inhibit the activity of the enzymes MAO. In general, imidazoline drugs were found to be weak MAO inhibitors and showed little discrimination between the two MAO isoforms (Ozaita et al., 1997). In contrast, competition binding assays using the selective radioligands [^{3}H]Ro41-1049 (MAO-A) and [^{3}H]Ro19-6327 (MAO-B) confirmed and extended previous findings demonstrating that some endogenous and exogenous β-carboline alkaloids are potent inhibitors of MAO (catalytic site) and more selective for MAO-A than MAO-B (Kim et al., 1997). On the other hand and as it was the case for imidazoline drugs (Ozaita et al., 1997), no significant relations were found, in general, when the potencies (K_i values) of the β-carbolines at the high-or the low-affinity site

of the imidazoline I_2 receptor labelled with [^{3}H]2-BFI were correlated with the corresponding drug potencies for inhibition of [^{3}H]Ro 41-1049 binding to MAO-A and [^{3}H]Ro 19-6327 binding to MAO-B in rat brain and liver membranes, indicating that imidazoline I_2 receptors are not related to the site of action of β-carbolines on MAO activity (see also Ozaita et al., 1997). However, one exception was noticed in the cerebral cortex (pK_i on MAO-A vs. pK_{iH} on I_2 sites, $r=-0.85$, $n=6$, $P=0.03$) the relevance of which is not readily apparent. On the other hand, it has been reported that I_2 sites in liver and brain are lost in MAO-B, but not MAO-A, deficient mice (Remaury et al., 2000), and that MAO inhibition by imidazoline ligands could occur through allosteric mechanisms (Raddatz et al., 2000). However, the in vivo alkylation of brain I_2 sites (>90%) with the selective and irreversible ligand BU99006 (5-isothiocyanato-2-benzofuranyl-2-imidazoline) had no effect on the activity of MAO-A and MAO-B (Paterson et al., 2003), further suggesting the existence of other imidazoline I_2 binding proteins that are distinct from the enzyme MAO (Ozaita et al., 1997; Paterson et al., 2003).

There are several evidences that suggest the existence of functional interactions between imidazoline I_2 ligands and β-carboline compounds with the opioid system in the rodent brain. Agmatine, a putative endogenous ligand for imidazoline receptors, has been shown to attenuate tolerance to morphine-induced antinociception in rats (Kolesnikov et al., 1996; Boronat et al., 1998). In addition, the concurrent chronic administration of morphine with the I_2 ligands idazoxan, 2-BFI, LSL 60101 or LSL 61122 (13 days) also prevented or attenuated the development of tolerance to the opiate, which was still apparent 6 days after discontinuation of the chronic treatment with LSL 60101-morphine (Boronat et al., 1998). On the other hand, the β-carbolines norharman, harman and harmine (Cappendijk et al., 1994; Aricioglu-Kartal et al., 2003; present results), the selective I_2 receptor ligands 2-BFI, BU224 and LSL 60101 (Hudson et al., 1999; present results) as well as agmatine (Aricioglu-Kartal and Uzbay, 1997) were shown to reduce significantly some of the symptoms of morphine withdrawal in rat models of opiate dependence. The molecular mechanisms implicated in the behavioural effects of these drugs could be associated, in part, with the attenuation of an increased activity of locus ceruleus neurones and their noradrenergic projections in the brain, which are mainly involved in the expression of the somatic symptoms of opiate withdrawal (review in Sastre-Coll et al., 2002). Accordingly, 2-BFI was demonstrated to reduce naloxone-induced hyperactivity (firing rate) of locus ceruleus neurones in morphine-dependent rats (Ruiz-Durántez et al., 2003). Also recently, agmatine was shown to attenuate the increased levels of cAMP (induced by naloxone) as well as the higher immunocontent of tyrosine hydroxylase (regulated by the cAMP system) in morphine-treated rat brain slices that included the locus ceruleus (Aricioglu et al. 2004). In the current study, the selective imidazoline I_2 ligand LSL 60101 and the β-carboline norharman blocked the stimulatory effect of naloxone on DOPA/noradrenaline synthesis (in vivo activity of tyrosine hydroxylase) in the cerebral cortex and hippocampus of morphine-dependent rats, which may contribute to the attenuated opiate-withdrawal reaction observed with these drugs. These results together suggest that some β-carbolines and selective imidazoline I_2 drugs may prove to be useful agents for the management of the opiate abstinence syndrome in humans.

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